MOLECULAR STRUCTURE OF THE OVALBUMIN GENE AND ITS GENOTYPIC ALLELES

(DNA cloning, intervening sequences, restriction mapping, electronmicroscopic mapping)

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INTRODUCTION

Mapping of DNA has been over the years within the domain of genetics rather than chemistry, mainly because of the long-standing difficulties involved in its sequence determination. DNA is chemically very monotonous, almost indistinguishable along its strand. However, this monotony of DNA was recently surmounted by the discovery of restriction endonucleases, which are enzymes that cleave DNA at precise sequences and give rise to chemically defined DNA fragments. The availability of such DNA fragments has opened the door for chemical methods of sequence determination. It was also soon realized that restriction DNA fragments could be ligated into recombinant DNA molecules with DNA vectors, such as plasmids or phage DNA, and propagated indefinitely in bacteria, a procedure called DNA cloning (COHEN *et al.*, 1973). Thus cloning of a specific DNA fragment, followed by analysis of its sequence, became the new strategy in attempts to gain further insight into the functioning of eukaryotic genes. Out of this technological triad of restriction enzyme analysis, molecular cloning and DNA sequencing has emerged an entirely new concept of eukaryotic gene architecture. The only prerequisite for such molecular studies of an eukaryotic gene is that we can obtain its messenger RNA. As messenger RNA could best be identified by the protein it coded for, now in turn, it is the most powerful reagent to identify and analyze genes.

Ovalbumin Messenger RNA

The complete sequence of chicken ovalbumin mRNA was determined by McREYNOLDS *et al.* (1978) and is presented in Fig. 1. It is 1,859 residues long, excluding its terminal 'cap' and poly A. The region coding for ovalbumin lies

close to the 'cap', but there is a leader sequence of 64 non-coding nucleotides preceding the coding residues at the 5'-end of the mRNA. The coding sequence of 1,158 nucleotides is separated from the poly A at the 3'-end by an extensive 3'-noncoding region of 637 nucleotides which may have no function that is precisely dependent on its sequence. Oval-bumin mRNA is thus more than 600 nucleotides longer than the minimum nucleotide number required to code for ovalbumin, a protein composed of 385 amino acids.

```
mG
|                                                      M  G  S  I  G  A  A  S  M  E  F  C  F  D  V  F  K  E  L
ACAUACAGCUAGAAAGCUGUAUUGCCUUUAGCAGUCAAGCUCGAAAGACAACUCAGAGUUCACCAUGGGCUCCAUCGGUGCAGCAAGCAUGGAAUUUUGUUUUGAUGUAUUCAAGGAGCU
         10        20        30        40        50        60        70        80        90       100       110       120

   K  V  H  H  A  N  E  N  I  F  Y  C  P  I  A  I  M  S  A  L  A  M  V  Y  L  G  A  K  D  S  T  R  T  Q  I  N  K  V  V  R
CAAAGUCCACCAUGCCAAUGAGAACAUCUUCUACUGCCCCAUUGCCAUCAUGUCAGCUCUAGCCAUGGUAUACCUGGGUGCAAAAGACAGCACCAGGACACAAAUAAAUAAAGGUUGUUGG
         130       140       150       160       170       180       190       200       210       220       230       240
                                                P
   F  D  K  L  P  G  F  G  D  S  I  E  A  Q  C  G  T  S  V  N  V  H  S  S  L  R  D  I  L  N  Q  I  T  K  P  N  D  V  Y  S
CUUUGAUAAACUUCCAGGAUUCGGAGACAGUAUUGAAGGCUCAGUGUGGCACAUCUGUAAACGGUUCACUCACUUAGAGACAUCCUCAACCAAAUACCAAACCAAAUGAUGUUUAUUC
         250       260       270       280       290       300'      310       320       330       340       350       360

   F  S  L  A  S  R  L  Y  A  E  E  R  Y  P  I  L  P  E  Y  L  Q  C  V  K  E  L  Y  R  G  G  L  E  P  I  N  F  Q  T  A  A
GUUCAGCCUUGCCAGUAGACUUUAUGCUGUAGAGAGAAGACUUAAUACCCAACCCUGCCAGAAUACUUGCAGUGUUGGUGAAGGAACUGUAUAGAGGAGGCUUUGGAACCUUAUCAACUUCAAACAGCUGC
         370       380       390       400       410       420       430       440       450       460       470       480

   D  Q  A  R  E  L  I  N  S  W  V  E  S  Q  T  N  G  I  I  R  N  V  L  Q  P  S  S  V  D  S  Q  T  A  M  V  L  V  N  A  I
AGAUCAAGCUCGAGAGACUGCAUCAAUUCCUGAUGGUAGAAGUCAGACAAAUGGAAUUAUCAGAAAAUGUCCUAGCCAAGCUCGUGGAUUCCCAAACUGCAAUGGUUCUUGUUAAUGCCAU
         490       500       510       520       530       540       550       560       570       580       590       600

   V  F  K  G  L  W  E  K  A  F  K  D  E  D  T  Q  A  M  P  F  R  V  T  E  Q  E  S  K  P  V  Q  M  M  Y  Q  I  G  L  F  R
UGUCUUCAAAGGACUGUGGGGAGAAAGCAUUUAAGGAUGAAGACACAAAGGCAAUGCCUUUCAGAGUUACAGAGUGAGCAAGAAAGCAAACCUGCAGAUGAUGUACCAGAUAGGUUUAUUUAG
         610       620       630       640       650       660       670       680       690       700       710       720

   V  A  S  M  A  S  E  K  M  K  I  L  E  L  P  F  A  S  G  T  M  S  M  L  V  L  L  P  D  E  V  S  G  L  E  Q  L  E  S  I
AGUGGCAACAUGGCUUCUGAGAAAAUGAAGAUCCUGGAGCUUCCAUUUGCCAGUGGGACAAUGAGCAUGUUGGUGCUGUUGCCUGAUGAAGUCUCAGGCCUUGAGCAGCUUGAGAGUAU
         730       740       750       760       770       780       790       800       810       820       830       840
                                                                                                        CHO
   I  N  F  E  K  L  T  E  W  T  S  S  N  V  M  E  E  R  K  I  K  V  Y  L  P  R  M  K  M  E  E  K  Y  N  L  T  S  V  L  M
AAUCAACAACUUUGAAAAACUGAAUGGACCCAGUUCUAAUGUUAUGGAAGAGAGGAAGAUCAAAGUGUACUUACCUCGCAUGAAGAUGGAGGAAAAAAUACAACCUCACAUCUGUCUUAAU
         850       860       870       880       890       900       910       920       930       940       950       960

   A  M  G  I  T  D  V  F  S  S  S  A  N  L  S  G  I  S  S  A  E  S  L  K  I  S  Q  A  V  H  A  A  H  A  E  I  N  E  A  G
GGCUAUGGGCAUUACUGACGUGUUUAGCUCAGCCAAUCUGUCUGGCAUCUCCUCAGCAGAGCCUGAAGAAAUCCAAGCUGUCCAUGCAGCACAUGCAGAAAUCAAUGAAGCAGG
         970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
                            P
   R  E  V  V  G  S  A  E  A  G  V  D  A  A  S  V  S  E  E  F  R  A  D  H  P  F  L  F  C  I  K  H  I  A  T  N  A  V  L  F
CAGAGAGGUGGUGAGGUCAGCAGAGGCCGGAGUGGACGCCAGCGGCUCUGAAGAAUUUAGGGCUGACCAUCCAUUCCUCUUCUGUAUCAAGCACAUCGCAACCAACGCCGUUCUCUUU
        1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200

   F  G  R  C  V  S  P  *
CUUUGGCAGAGUGUGUUCCUCCUUAAAAAGAAGAAAGCUGAAAAACUCUGUCCUUCCAACAAGACCCAGAGCACUGUAGUAUCAGGGGUAAAAUGAAAGUAUGUUAUUCUGCUGCAUCCA
        1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320

GACUUCAUAAAAGCUGGAGCUUAAUCUAGAAAAAAAAACAGAAAGAAAUUACACUGUAGAAACAGGGUGCAAUUCACUUUUCCUUUACACAGAGUAAAUACUGGUAAACUCAUGGAUGAAGGC
        1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440

UUAAGGGGAAUGAAAUUGGAGUCAUCACACUGUGAAAAAUGCAACCUGAUACAUCAGCAGAAGGUUUAUUGGGGGAAAAAUGCAGCCUGUCCAAUUUAAGCCAGAAUAUCUGUAUGACCAAGCUGUCU
        1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560

CCAGAAUUUAGCUCACUCAAAAUCUCUCAGAUUAAUAUUAUCAACUGUCACCAACCAUUCCUAUGCUGACAAGGCUGUCUUGUCUGGUGUUCCUGAUACUACAAGGCUCUUCCUGACUUU
        1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680

CCUAAAGAUGCAUUAUAAUAAAAAUCUUAUAAAAUUCACAUUUCUCCUGUCUUUCUCUUAUGCAAUCAUUGGUGGCAAAUAUGGUAUAUUCUCAUCUUUAGUAUUAGAUUUCUUGUACCCUAUGU
        1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800

AAUGGGGUCUUGUGAAUGUGCUCUUUUGUUCCUUUAAUCAUUAUAAUAUAAAAACAUGUUUUAAGC - Poly A
        1810      1820      1830      1840      1850
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Fig. 1 - Sequence of ovalbumin messenger RNA. Residues 13-1,859 were present in the plasmid pOV230; residues 1-12 and 1,731-1,859 were sequenced directly from the mRNA. * Marks the termination codon, P the serine phosphate residues, and 'CHO' the carbohydrate. Mature ovalbumin lacks the N-termi-nal methionine; the resulting N-terminal glycine is N-acetylated. The amino acid composition, excluding the ini-tiation Met, is Ala (A, 35; Arg (R), 15; Asn (N), 17; Asp (D), 14; Cys (C), 6; Glu (Q), 15; Glu (E), 33; Gly (G), 19; His (H), 7; Ile (I), 25; Leu (L), 32; Lys (K), 20; Met (M), 16; Phe (F), 20; Pro (P), 14; Ser (S), 38; Thr (T), 15; Trp

(W), 3; Tyr (Y), 10; and Val (V), 31. Total, 385 residues.
(McREYNOLDS *et al.*, 1978).

The Recombinant Plasmid pOV230

Although ovalbumin messenger RNA of relatively high
purity was obtained (ROSEN *et al.*, 1975; WOO *et al.*, 1975),
it is more convenient to propagate its double-stranded com-
plementary DNA copy in a plasmid rather than to rely on the
availability of the mRNA. First of all, eukaryotic mRNA can
not be obtained at 100 percent purity and secondly, it is
difficult to obtain large quantities. However, once cloned
in a recombinant plasmid, unlimited quantities of highly pure
DNA become available. A full-length duplex DNA copy of oval-
bumin mRNA was therefore synthesized and cloned (McREYNOLDS
et al., 1977) in the *Escherichia coli* plasmid pMB9. The oval-
bumin DNA in this recombinant plasmid (pOV230) contains a
copy of all but the terminal 12 bases present at the 5'-end
of $mRNA_{OV}$. It contains 52 base pairs of DNA preceding the
initiation codon AUG, the entire peptide coding region for
ovalbumin and 634 nucleotide pairs of untranslated sequences
following the termination codon UAA. The ovalbumin DNA,
recovered after excision from the recombinant plasmid, can
now be labeled with $[^{32}P]$ by nick-translation (MANIATIS *et
al.*, 1975) and used as a specific probe for ovalbumin DNA
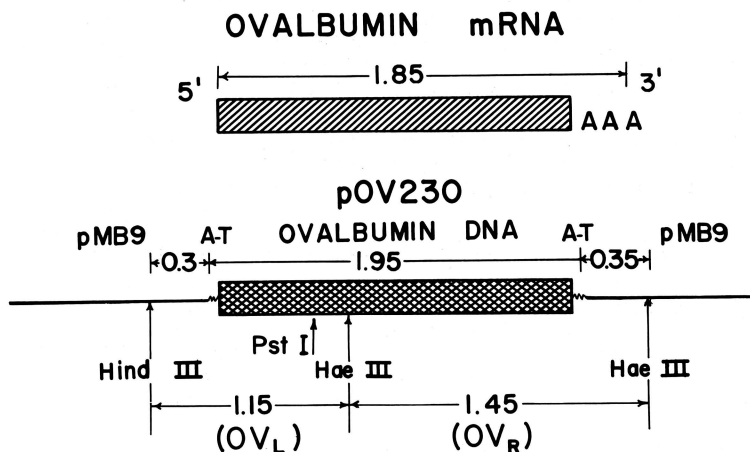sequences to map and clone the ovalbumin gene.

Fig. 2 - Orientation of the full-length double-s-randed
DNA_{OV} insert in the recombinant plasmid pOV230. The sites
of cleavage by *Hae* III and *Hind* III are indicated by arrows,
and the numbers represent the restriction fragments sizes

in kilobases.

Restriction Mapping of the Ovalbumin Gene

Our initial attempts to clone the natural ovalbumin gene called for an enrichment of DNA containing ovalbumin sequences. To this end, total chicken DNA was digested with the restriction endonuclease Eco RI, separated according to size by agarose gel electrophoresis and transferred to nitrocellulose paper according to the method of SOUTHERN (1975). The filter was then hybridized with a radioactive probe derived from the recombinant plasmid pOV230 (Fig. 2), followed by autoradiography. Since there are no Eco RI cleavage sites within the ovalbumin structural gene (McREYNOLDS et al., 1978), all of the ovalbumin gene sequences should be found in DNA fragments of one size. To our surprise however, at least three DNA fragments were found that contained ovalbumin sequences. These were 9.5 kilobases (later determined more precisely as 9.2 Kb) 2.4 Kb and 1.8 Kb in size; a faint 1.3 Kb DNA fragment was frequently also observed.

The chimeric plasmid pOV230 was subsequently digested with the restriction endonucleases Hae III and Hind III to yield two DNA fragments containing the left (OV_L) and right (OV_R) halves of the ovalbumin DNA insert (Fig. 2). These two fragments were separated by agarose gel electrophoresis, labeled with [^{32}P] by nick-translation (MANIATIS et al., 1975) and employed again as specific hybridization probes to identify the Eco RI ovalbumin DNA fragments derived from chromosomal DNA. The 9.2 Kb DNA hybridized only with the OV_R probe, whereas the remaining 2.4 Kb, 1.8 Kb and 1.3 Kb DNA fragments formed a hybrid only with OV_L probe (Fig. 3). This result was consistent with the interpretation that within the native ovalbumin gene there were at least two non-structural (intervening) sequences that were cleaved by Eco RI. Similar experiments were carried out with total chicken DNA digested with a variety of restriction endonucleases and the resulting DNA fragments containing ovalbumin structural gene sequences were measured (Fig. 3). From these results we obtained a preliminary restriction map of the native ovalbumin gene with the locations of structural and intervening DNA sequences within this gene (Fig. 4). Similar results were reported by Doel et al. (1977), Breathnack et al. and Weinstock et al (1978). The three Eco RI DNA fragments are thus arranged in the order (5')-2.4-1.8-9.2-(3') within the ovalbumin gene.

Cloning of Fragments of the Ovalbumin Gene

The Eco RI 2.4, 1.8 and 9.2 Kb fragments of the ovalbumin gene were enriched about 200-fold and separated from one another by a combination of RPC-5 column chromatography (HARDIES and WELLS,1976) and gel electrophoresis. The enriched 2.4 Kb DNA was cloned (WOO et al., 1978) using the certified EK2 vector λgtWES·λB (LEDER et al., 1977). One

out of approximately 4,000 recombinant phage plaques
hybridized with the [^{32}P] labeled probe derived from pOV230
which contained the entire ovalbumin structural gene. The
1.8 Kb fragment was cloned similarly (DUGAICZYK *et al.*,
1978) using also λgtWES·λb as the cloning vector. The 9.2
Kb fragment was subsequently cloned (DUGAICZYK *et al.*,
1979) using as cloning vector the λ phage Charon 4A (BLATTNER
et al., 1977) which accepts larger DNA fragments. As pre-
dicted earlier (LAI *et al.*, 1978) and 9.2 Kb fragment con-
tains the 3'-end of the ovalbumin gene. Although the λ
phage is a very efficient cloning system, it is not suited
for the production of large quantities of DNA. Therefore,
the insert DNA was cleaved from their respective recombinant
phage DNA and recloned in the plasmid vector pBR322 (BOLIVAR
et al., 1977) which produces higher yields of cloned DNA.

Structure of the Ovalbumin Gene

     The cloned *Eco* RI DNA fragments of the ovalbumin gene
were recovered from their recombinant plasmids. They were
then studied by hybridization with ovalbumin mRNA followed
by electron microscopy of the resulting hybrids, by restric-
tion enzyme mapping and by DNA sequence analysis (DUGAICZYK
et al., 1978). The results revealed the existence of seven
intervening sequences within the ovalbumin gene instead of
two, as previously concluded from studies on genomic DNA.
A more precise restriction map of the ovalbumin gene was
constructed (DUGAICZYK *et al.*, 1978) localizing the distri-
bution of the intervening and structural sequences within
this gene. More importantly, however, it was concluded
that the 5'-terminus of the ovalbumin gene was not located
within the cloned 2.4 Kb DNA fragment. Direct sequence
analysis indicated that the 2.4 Kb fragment contained the
ovalbumin sequence starting from nucleotide 46 of the struc-
tural gene (ROBERTSON *et al.*, 1978). In a search for the
5'-end of the ovalbumin gene containing the missing 45
nucleotides of the ovalbumin messenger RNA, we have cloned
additional overlapping restriction DNA fragments from geno-
mic chicken DNA (DUGAICZYK *et al.*, 1979). An *Hin*d III 3.2
Kb fragment and a *Pst* I 4.5 Kb fragment were cloned, both of
which overlap in part with the *Eco* RI 2.4 Kb fragment, but
extend at their 5'-termini further into genomic DNA. The
presence of the missing structural gene sequence was at
last verified within the *Pst* I 4.5 Kb DNA, which permitted
us to construct a complete physical map of the ovalbumin
gene (DUGAICZYK *et al.*, 1979) (Fig. 5).

     Subsequently, we obtained from Drs. Axel, Parker and
Hodgson a clone containing the entire ovalbumin gene from
a chick gene library generated by the method of LAWN *et al.*
1979). Analyses of this cloned DNA by restriction and
electronmicroscopic mapping confirmed the location of the
5'-end of the gene as shown in Fig. 5 as well as the
existence of seven intervening sequences within the oval-
bumin gene (Fig. 6) (DUGAICZYK *et al.*, 1979). Similar
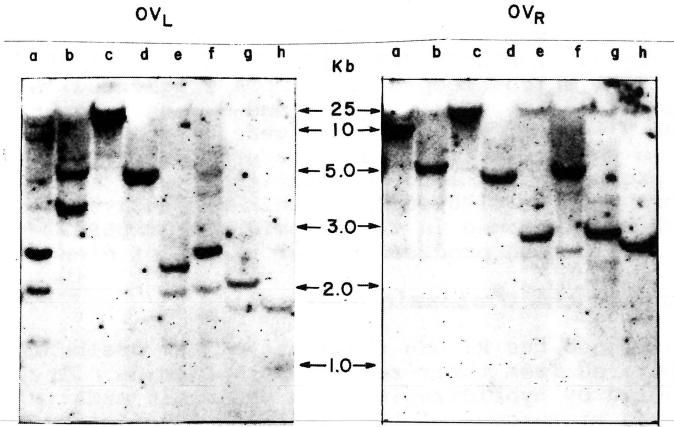results were reported from other laboratories, except that

Fig. 3 - Gel electrophoretic separation of the products of digestion of total chicken DNA with various restriction endonucleases. Lanes: a, *Eco* RI; b, *Hind* III; c, *Bam* HI; d, *Pst* I; e, *Eco* RI plus *Hind* III; f, *Eco* RI plus *Bam* HI; g, *Eco* RI plus *Pst* I; and h, *Eco* RI plus *Hae* III. The autoradiograms of the gels show hybridization to the OV_L (left) and the OV_R (right) probes.
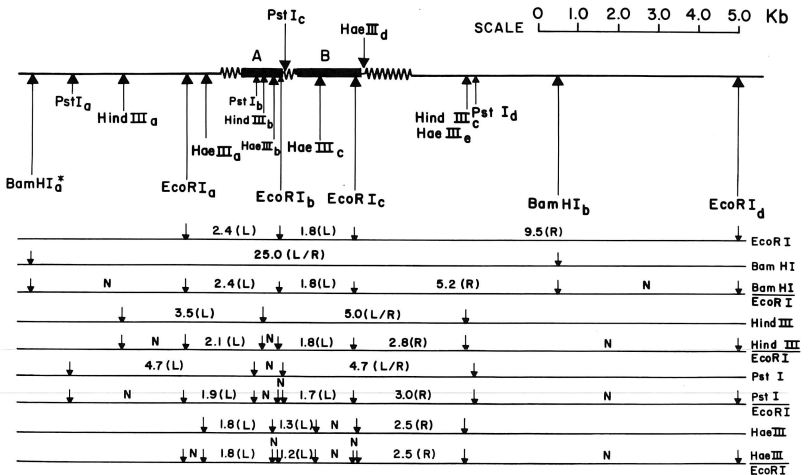
only six intervening sequences were concluded in this gene
(GARAPIN *et al.*, 1978; MANDEL *et al.*, 1978).
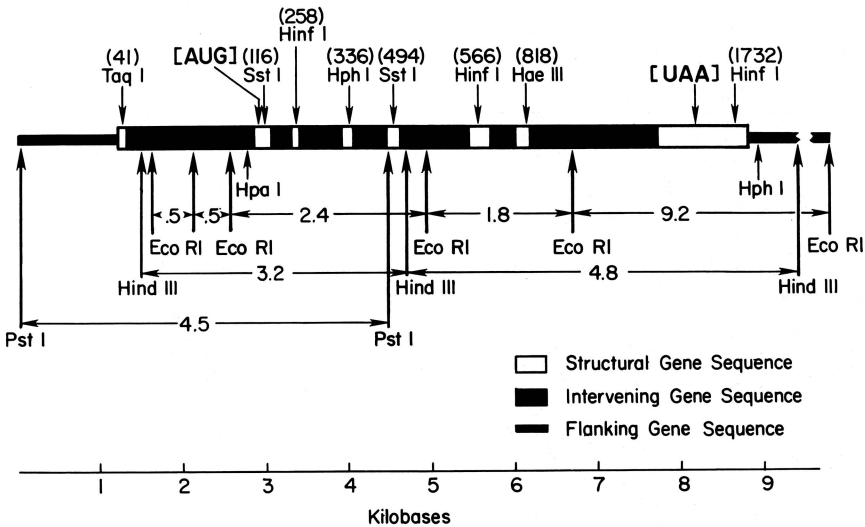
Fig. 5 - Physical map of the entire ovalbumin gene dis-
playing some of the key restriction sites, the locations
of the initiation and termination codons, and regions of
structural and intervening sequences. This map is con-
structed from analyses of gene fragments cloned in our
laboratory. They are: 2.4 Kb *Eco* RI DNA; 1.8 Kb *Eco* RI
DNA; 9.2 Kb *Eco* RI DNA; 3.2 Kb *Hind* III DNA; and 4.5 Kb
Pst I DNA.

←

Fig. 4 - Model for the organization of the ovalbumin gene
obtained from restriction mapping of genomic chicken DNA.
Structural DNA sequences, present in mRNA, are represented
by ᴍᴡ ; intervening DNA sequences are represented by ▬ ;
and flanking DNA sequences are represented by — . Various
sites are shown by arrows; those above the line are restric-
tion sequences present in ovalbumin mRNA. The sizes (in
kilobases) of different restriction fragments are shown
below the model. (L), (R) and (L/R) indicate that the DNA
fragment was detected by hybridization with OV_L, OV_R or
both OV_L and OV_R probes, respectively. (N) represents DNA
fragments that did not hybridize to any one of the above
probes because of lack of sequence homology and were not
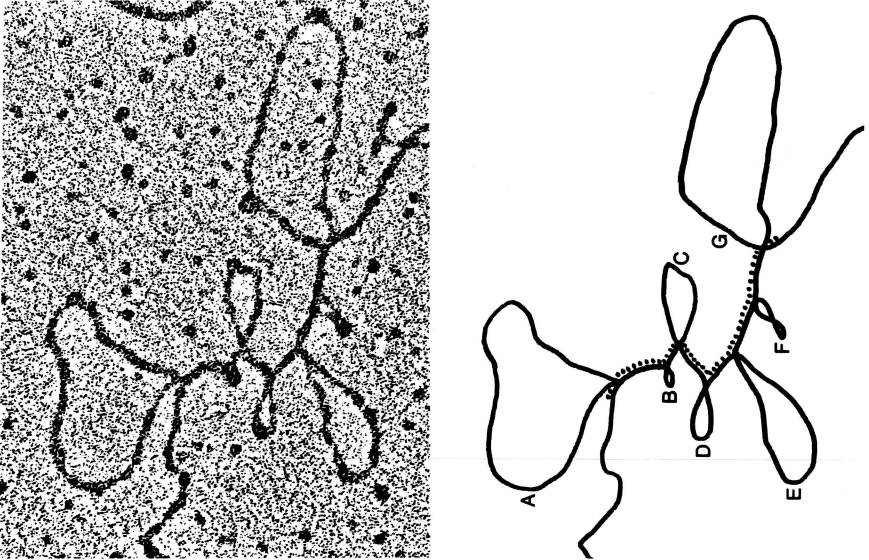detected (LAI *et al.*, 1978).

Fig. 6 - Electron micrograph and line drawing of a hybrid
molecule formed between the ovalbumin gene and ovalbumin
mRNA. ___ represents DNA, --- represents ovalbumin mRNA.
Loops A, B, C, D, E, F and G represent the seven interven-
ing DNA sequences (See Fig. 5) that have no sequence homol-
ogy with mature ovalbumin mRNA (DUGAICZYK *et al.*, 1979).

Genotypic Alleles

It has been hypothesized that the 1.3 Kb *Eco* RI frag-
ment may be allelic with the 1.8 Kb DNA (WEINSTOCK *et al.*,
1978). The hypothesis gained support from our initial exper-
iments showing that the cloned 1.8 Kb DNA would hybridize
with both 1,8 Kb and 1.3 Kb *Eco* RI fragments derived from
genomic chicken DNA (DUGAICZYK *et al.*, 1978). Subsequent
cloning and detailed restriction mapping and partial sequence
determination demonstrated that the two DNA fragments were
indeed genotypic alleles (LAI *et al.*, 1979). In one of the
allelic forms an extra *Eco* RI restriction site occurs within
one of the intervening sequences of the ovalbumin gene.
Otherwise the two DNA fragments have complete sequence
homology (Fig. 7).

A comparison of nucleotide sequences of the 1.8 Kb DNA
(CATTERALL *et al.*, 1979) and the 1.3 Kb fragment (LAI *et al.*,
1979) is given below.

```
5'-OV1.8      ...CCCATAAATTCCTACATTCTCTCTATCTACCTTGTGCTTG...
5'-OV1.3         AATTCCTACATTCTCTCTATCTACCTTGTGCTTG...
```
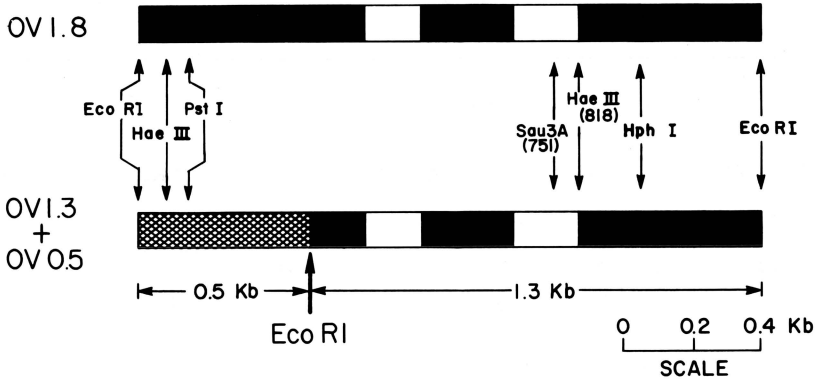

Fig. 7 - Restriction endonuclease maps of the cloned OV1.8 and OV1.3 Kb DNA. Black sections denote intervening sequences, white sections represent ovalbumin structural DNA. The cross-hatched region represents the intervening sequence comprising the 0.5 Kb DNA fragments (LAI *et al.*, 1979).

The 5'-sequence of OV1.3 starts with nucleotides AATTC, revealing part of an *Eco* RI site. In OV1.8, the nucleotide 522, which precedes this sequence is an (A). Thus a single mutation in the hexanucleotide "AAATTC" to give "GAATTC" will create the additional *Eco* RI restriction site, giving rise to two *Eco* RI fragments of 0.5 Kb and 1.3 Kb in place of the intact 1.8 Kb DNA within the chicken genome.

Frequency of Occurrence of the Two Alleles

Total DNA extracted from individual hens and roosters was digested with *Eco* RI, separated by gel electrophoresis, and allowed to hybridize with the labeled 1.8 Kb probe (LAI *et al.*, 1979). Of the twenty chickens tested, eight possessed only the 1.8 Kb DNA. Thus 40% of the White Leghorn population appears to be homozygous for the 1.8 Kb DNA and does not contain the extra *Eco* RI site within its ovalbumin gene. Only two animals possessed the 1.3 Kb and the 0.5 Kb DNA. Therefore only 10% of the population is homozygous for the 1.3 Kb DNA, containing the extra *Eco* RI site within the 1.8 Kb DNA of the ovalbumin gene. As expected, the 0.5 Kb fragment was found to be present only in association with the 1.3 Kb fragment, confirming that they are both derived from the 1.8 Kb DNA. The remaining ten chickens had all three (1.8, 1.3 and 0.5 Kb) DNA fragments. Thus,

50% of the population is heterozygous for this extra *Eco* RI
restriction site. The exact nature of these alleles must
await further analyses; they do follow Mendelian segre-
gation upon mating (R. AXEL, personal communication).

Additional experiments were also performed on indivi-
dual chickens, using other restriction endonucleases to
analyze the patterns of restriction fragments derived from
the ovalbumin gene (LAI *et al.*, 1979) as a result, a similar
allelic variation was found with respect to *Hae* III restric-
tion sites located within intervening sequences of the
ovalbumin gene of individual chickens.

## Comments on the Sequence Organization of the Ovalbumin Gene

Despite the intricate sequence organization of the
ovalbumin gene (Fig. 5), a correct structural sequence may
be obtained from left to right by omitting the intervening
sequences. During expression, the gene is transcribed into
a high molecular weight RNA containing all the intervening
sequences (ROOP *et al.*, 1978). This primary RNA transcript
appears to be processed subsequently into the mature
messenger RNA by excision of the intervening sequences and
proper ligation of the structural sequences. The presently
described mutations within the intervening sequences will
not be represented in the mature mRNA and the protein
itself as long as they do not interfere with processing
of the intervening sequences. Such genotypic alleles have
no apparent phenotypic manifestations and they differ from
classic silent mutations in that the latter occur within
structural gene sequences. Their existence can be revealed
only by molecular cloning and sequence analysis of the natu-
ral gene. Of further interest in the case of the ovalbumin
gene may be the fact that over 8,000 base pairs of DNA
sequences are required to code for a mature mRNA of 1,859
bases which in turn codes for a protein composed of 385
amino acids. Thus, storage of genetic information appears
to require a greater complexity than what is contained in
the functional biologic products.

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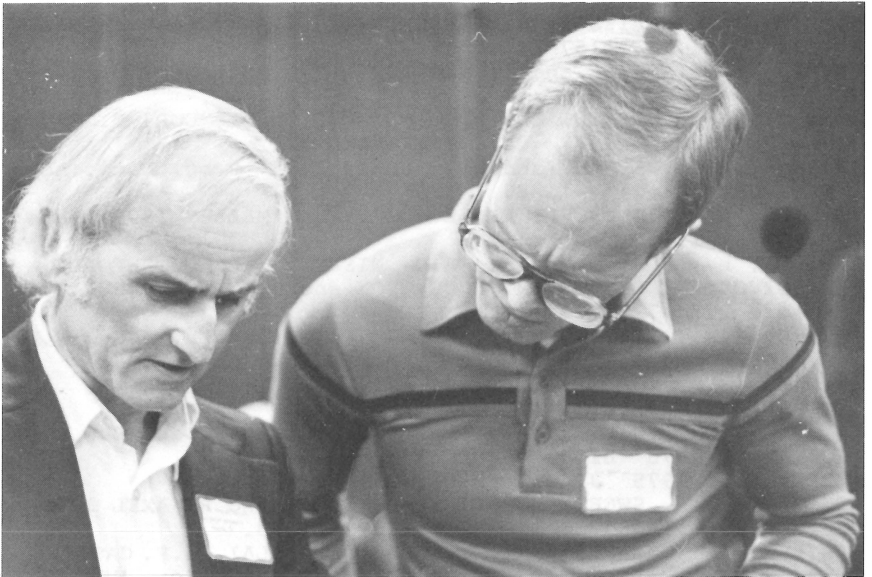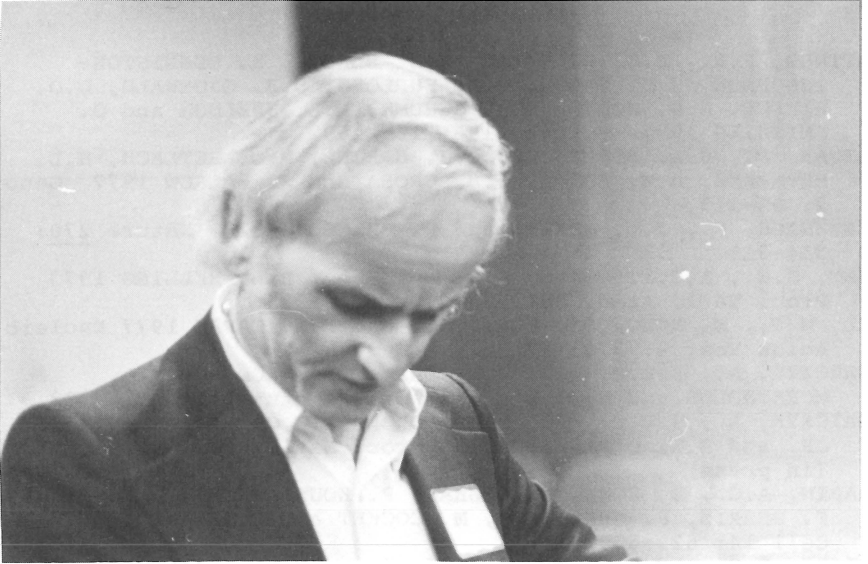
Dr. Dugaiczyk (top and lower left). Graduate student Jim Worstell looks on.